



The  
Patent  
Office  
09/171671

GB 97/01153

PRIORITY DOCUMENT

The Patent Office  
Cardiff Road  
Newport  
Gwent  
NP9 1RH

REC'D 26 MAY 1997

ISO PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation and Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

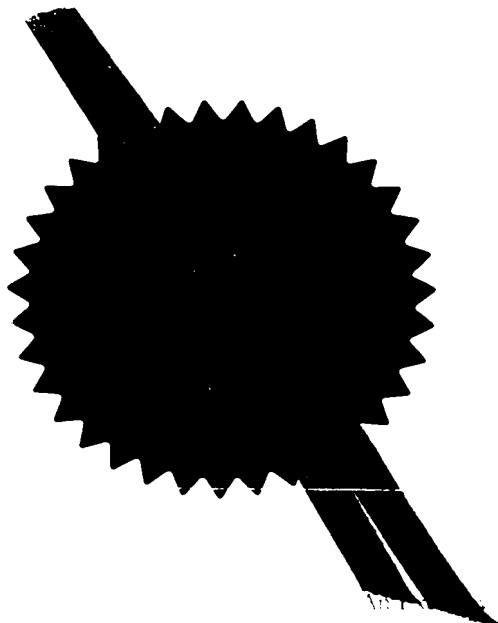
In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

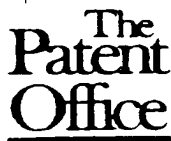
Dated

13 MAY 1997





Patents Form 1/77



Patents Act 1977  
(Rule 16)

**Request for grant of a patent**

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form.)

The Patent Office

Cardiff Road  
Newport  
Gwent NP9 1RH

Fee: £25


1. Your reference	37520(3)/JMD		
2. Patent application number (The Patent Office will fill in this part)	<b>9624584.0</b>		<b>27 NOV 1996</b>
3. Full name, address and postcode of the or of each applicant ( <u>underline all surnames</u> )	PEPTIDE THERAPEUTICS LIMITED 321, Cambridge Science Park, Milton Road, Cambridge CB4 4WG.		
Patents ADP number (if you know it)			
If the applicant is a corporate body, give the country/state of incorporation	United Kindom		
4. Title of the invention	Substrates and Inhibitors of Proteolytic Enzymes		
5. Full name, address and postcode in the United Kingdom to which all correspondence relating to this form and translation should be sent	Reddie & Grose 16 Theobalds Road LONDON WC1X 8PL		
Patents ADP number (if you know it)	91001 /		
6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number	Country	Priority application (If you know it)	Date of filing (day month year)
7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing (day month year)	
8. Is a statement of inventorship and of right to grant of a patent required in support of			

*(If the applicant is a corporate body, see note (d))*

## Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document.

Continuation sheets of this form

 Description 57  
Claim(s) 6  
Abstract 0  
Drawing(s) 16

10. If you are also filing any of the following, state how many against each item.

Priority documents 0  
Translations of priority documents 0  
Statement of inventorship and right to grant of a patent (*Patents Form 7/77*) 0  
Request for preliminary examination and search (*Patents Form 9/77*) 0  
Request for substantive examination (*Patents Form 10/77*) 0  
Any other documents (please specify)

11. I/We request the grant of a patent on the basis of this application.

Signature

Date



26 November 199

12. Name and daytime telephone number of person to contact in the United Kingdom

J M DAVIES  
0171-242 0901

### Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or such direction has been revoked.

### Notes

- If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.
- Write your answers in capital letters using black ink or you may type them.
- If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- Once you have filled in the form you must remember to sign and date it.
- For details of the fee and ways to pay please contact the Patent Office.

Substrates And Inhibitors of Proteolytic Enzymes

The present invention relates to the field of compounds  
which are substrates or inhibitors of proteolytic enzymes  
5 and to apparatus and methods for identifying substrates or  
inhibitors for proteolytic enzymes.

Many therapeutically useful drugs act as enzyme  
inhibitors. In particular, proteolytic enzyme inhibitors  
have been the focus of much attention in the  
10 pharmaceutical industry, because they play a variety of  
roles in a multitude of biological systems. Their  
proteolytic activities are related to processes ranging  
from cell invasion associated with metastatic cancer to  
evasion of an immune response, as seen in certain  
15 parasitic organisms; from nutrition to intracellular  
signalling to the site-specific proteolysis of viral  
proteases and eukaryotic hormone-processing enzymes.  
However, the traditional random screening methods for the  
identification of lead molecules as inhibitors of  
20 proteolytic enzymes are often laborious and time-  
consuming. Therefore new and efficient methods which can  
accelerate the drug discovery process are greatly in  
demand.

We consider that proteases contain an active catalytic  
25 site which tends to become increasingly activated as the  
recognition pockets' ( $S_1$  and  $S_2$  etc) and ( $S_1'$  and  $S_2'$  etc)  
become better occupied. Therefore, it is important that

inhibitors. Therefore, we have devised a combinatorial method for the rapid identification of these binding motifs which will greatly expedite the synthesis of inhibitors of a variety of proteolytic enzymes such as aspartyl proteases, serine proteases, metallo proteases and cysteinyl proteases.

The use of a fluorescence resonance energy transfer (FRET) substrate for the analysis of proteolytic enzyme specificity was first published by Carmel.<sup>2</sup> Since then many different quenched fluorogenic substrates for measuring enzyme inhibition have been described in the literature.<sup>4-11</sup> These substrates contain a fluorophore, F, in a P position (*vide supra*), which is quenched by another group, Q, present in a P' position (*vide supra*) and separated from F by the scissile bond. The advantage of the positioning of these residues, F and Q, is that cleavage of a peptide bond occurs between the two natural residues and, therefore, represents a more natural hydrolytic event rather than the cleavage and release of a C-terminal chromophore.

For example, Bratovanova and Petkov<sup>12</sup> have synthesised fluorogenic substrates from peptide 4-nitroanilides. N-acylation of peptide 4-nitroanilides with the aminobenzoyl (ABz) group yielded substrates that are internally quenched by the presence of the 4-nitroanilide moiety. Upon hydrolysis of the aminoacyl-4-nitroanilide bond, the highly fluorescent N-ABz group is released attached either to an amino acid or peptide.

Immobilised libraries, where substrates are attached to a polymer or biopolymer support, have also been used for mapping protease binding sites.<sup>13</sup> Singh et al. reported recently that enzymatic substrate activity of 38 selected octapeptides attached via a linker to controlled pore glass is predictive of the same activity of similar peptides in solution. However, these results are preliminary and only for a specific example. Therefore, it is not clear whether immobilised substrates attached to polymers can reliably replace soluble substrates in mapping the hindered protease binding sites, especially since the hydrophilic or lipophilic nature of the polymer and the size of the interstices within the polymer are bound to influence the reaction between the enzyme and its substrates.

Mixtures of internally quenched, fluorogenic substrates have also recently been described in which the quencher group, Q, is 2,4-dinitrophenyl (Dnp) and is attached to the P side of the scissile bond, while the fluorogenic group, is N-methyl anthranilic acid (Nma) and is attached to the P' side.<sup>14</sup>

The specificity of soluble peptide libraries have been determined.<sup>15,16</sup> Berman et al. described<sup>16</sup> an HPLC mass spectrometry technique in which 6 mixtures of 128 peptides were synthesised which were N-terminally labelled with the Dnp group in order to allow UV monitoring on the HPLC. The disadvantage of this approach is that each assay mixture

concentration of each separate component is limited by the size of the mixture because of overall solubility factors.

Drevin et al.<sup>8</sup> have suggested the use of individually synthesised fluorogenic substrates for the determination of enzyme activity using a chromophore which chelates lanthanide ions. Garmann and Phillips have suggested the use of FRET substrates in which the fluorogenic and quencher moieties are attached via thiol or amino functional groups after the peptide has been synthesised, but this has the disadvantage that they are not in library form and that these functional amino and thiol groups need to be selectively revealed after the peptide has been synthesised. Wang et al. have suggested the use of the EDANS and DABCYL fluoescor and quencher pairing for the individual synthesis of substrates for proteolytic enzymes.

The above methods which have used FRET techniques for the mapping of the active site around a specific protease suffer from one or more of the following disadvantages:

- i. because of general aqueous insolubility they do not produce mixtures of compounds in a form suitable for high throughput screening in aqueous solution.
- ii. the derivatised compounds cannot be prepared in combinatorial library form using solid phase techniques.
- iii. the mixtures which have been used<sup>8,9</sup> were not self-decoding, and needed time-consuming deconvolutive resynthesis for identification of the active molecules.



### Brief Description of the Invention

The present invention relates to the field of:

- i. Compounds which are substrates or inhibitors of proteolytic enzymes.
- 5 ii. Apparatus and methods which provide the rapid generation of structure-activity relationships using auto-deconvoluting combinatorial libraries, which facilitate the invention of novel inhibitors of proteolytic enzymes.
- 10 iii. Apparatus and methods which provide the detection and measurement of proteolytic enzyme activity using combinatorial FRET (fluorescence resonance energy transfer) libraries of molecules.
- 15 iv. Apparatus and methods which provide the establishment of biological assays for proteolytic enzymes through the rapid discovery of highly active substrates for proteolytic enzymes.

20 We describe herein apparatus and methods which can be used for the rapid generation of structure-activity relationship (SAR) data and, therefore, the characterisation of the binding motif of any protease, and which will, therefore, facilitate:

- 25 i. the development of a sensitive enzyme inhibition

- ii. the invention of novel compounds which are proteolytic enzyme inhibitors by rapid characterisation of the best binding motif.
- iii. computer aided drug design to design potent inhibitors using known methodology, and also in prioritising which pre-synthesised compounds in the in-house and commercially available databases to assay.

In a first aspect the invention provides novel compounds represented by the formula A-B-C-D-nE-F [I] in which; A represents a fluorescor internally quenched by F; B, C, D, and E represent groups such that the scissile bond between any two of these groups is a suitable bond; F represents a quencher capable of internally quenching the fluorescor A; and n represents an integer between 1 and 4 inclusive.

In some embodiments the suitable bond is an unsubstituted amide bond (see Example 1); in other embodiments the suitable bond is an ester bond (see Example 2).

In a second aspect the invention provides a combinatorial library of FRET compounds comprising a mixture of compounds of formula [I].

In a third aspect the invention provides for the use of such a combinatorial FRET library in a method which provides rapid generation of structure-activity relationships (SAR) which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET

(fluorescence resonance energy transfer) molecules to find a substrate or substrates for the enzyme. According to this method an identified substrate can be synthesised and used in biological assay for proteolytic enzymes. Novel  
5 substrates are included in the scope of the invention.

In a forth aspect the invention provides for the use of such a combinatorial FRET library in a method for detection and measurement of proteolytic enzyme activity against compounds of the library.

10 In a fifth aspect the invention provides a method which comprises the identification of an enzyme inhibitor or inhibitors wherein a FRET compound which has been identified as a substrate is used in an inhibition assay with the enzyme separately against a panel of possible  
15 inhibitors.

In a sixth aspect the invention provides a set of compounds which comprises two complementary FRET compound libraries. Such a set will be referred to hereafter as "apparatus" because it allows for the screening or assay  
20 method for identifying substrates or inhibitors of proteolytic enzymes. This set of compounds constituting an apparatus is capable of providing an auto-deconvoluting combinatorial library as will be described below.

In a seventh aspect the invention provides a method of  
25 identifying and synthesising an inhibitor of a proteolytic

energy transfer, molecules, deconvoluting the library to  
find a substrate or substrates for the enzyme and  
synthesis of an inhibitor based on the substrate or  
substrates. The direct product of this method is one or  
5 more novel proteolytic enzyme inhibitors.

In an eighth aspect the invention provides an inhibition  
assay which uses a FRET molecule, which has been  
identified as a substrate for the enzyme, wherein the  
molecule is assayed with the enzyme separately against a  
10 panel of possible inhibitors.

In a ninth aspect the invention provides a complementary  
pair of compound libraries L1 and L2 which constitute a  
set containing compounds of formula:

Aa-Bb-Cc-Dd-n(Ee)-Ff-Gg

15 giving  $a \times b \times c \times d \times e \times f \times g = M_n$  compounds in each  
library, there being a predetermined number (P1, P2) of  
mixtures each consisting of a predetermined number (Q1,  
Q2) of individual identifiable compounds in each library,  
wherein both L1 and L2 contain the same  $M_n$  compounds, but  
20 wherein any two compounds which are found together in one  
mixture of Q1 compounds of L1 are not found together in  
any one of the P2 mixtures of L2.

In a tenth aspect the invention provides a method of  
screening for enzymic activity using the libraries L1, L2  
25 described above in which the P1 mixtures of L1 and the P2  
mixtures of L2 are each placed separately into individual  
wells of two well plates, the well plates having wells

arranged in a format adapted to allow deduction of a unique active compound formula from the presence of activity in one well of the well plate of L1 and one well of the well plate of L2.

5 The apparatus of the invention preferably comprises two complementary compound libraries, L1 and L2, each containing  $n \times 1600$  compounds of the invention, of the type  $A-B_{1-10}-C_{1-10}-D_{1-8}-n(E_{1-2})-F-G$  [II], in which:

10 A = a fluoescor internally quenched by F, preferably an unsubstituted or substituted anthranilic acid derivative, connected by an amide bond to B

B, C, D, E, are natural or unnatural amino acid residues connected together by suitable bonds, although B, C, D and E can be any set of groups, provided that the scissile  
15 bond between D-E is an unsubstituted bond.

F = a quencher capable of internally quenching the fluoescor A, preferably an unsubstituted or substituted 3-nitrotyrosine derivative.

G = optionally present and is a hydrophilic moiety, preferably an aspartyl amide moiety. If present, G  
20 advantageously ensures that all compounds in the library are imparted with aqueous solubility. Also, G should not be a substrate for any type of enzyme.

n = any integer between 1 and 4 inclusive.

25 In an alternative, the scissile bond could be between B-C or C-D.

(Note that A and F herein correspond generally and respectively to moieties F and Q of the prior art referred to above).

The numbers represented in subscript following residues B, C, D and E refer to the number of possibilities from which those residues are selected. Thus, by way of illustrative example, A-B<sub>1-5</sub>-C-D-E<sub>1-2</sub>-F-G represents a mixture of the following ten compounds:

A-E<sub>1</sub>-C-D-E<sub>1</sub>-F-G  
10 A-B<sub>2</sub>-C-D-E<sub>1</sub>-F-G  
A-B<sub>3</sub>-C-D-E<sub>1</sub>-F-G  
A-B<sub>4</sub>-C-D-E<sub>1</sub>-F-G  
A-B<sub>5</sub>-C-D-E<sub>1</sub>-F-G  
A-B<sub>1</sub>-C-D-E<sub>2</sub>-F-G  
15 A-B<sub>2</sub>-C-D-E<sub>2</sub>-F-G  
A-B<sub>3</sub>-C-D-E<sub>2</sub>-F-G  
A-B<sub>4</sub>-C-D-E<sub>2</sub>-F-G  
A-B<sub>5</sub>-C-D-E<sub>2</sub>-F-G

The general combinatorial formula for each library can be expressed as:

$$A_1-B_{1-5}-C_{1-2}-D_8-n(E_2)-F_1-G_1 \quad [III]$$

providing  $1 \times 10 \times 10 \times 8 \times n \times 2 \times 1 \times 1 = 1600n$  compounds.

Both compound libraries, L1 and L2, of the above type are synthesized using solid phase techniques using the Multipin approach<sup>24</sup> such that each library contains 1600n compounds as 80n mixtures of 20 distinct, identifiable compounds. These 20 component mixtures are then placed

separately into each of 80 wells of a 96 well plate (the other two lanes are used for control experiments) and then screened against a known quantity of the protease.

Thus it is an important part of the invention that  
5 regardless of the number of compounds contained in the two libraries L1 and L2 ( $1600n$ , where  $n$  = any integer between 1 and 4) the libraries themselves are complementary and amenable to deconvolution without recourse to resynthesis. It is also an important part of the invention that the  
10 library matrix has been especially formatted so that the most important site pairings  $P_2$  and  $P_1$  for proteolytic enzymes can be identified immediately without recourse to resynthesis.

Those compounds of the type A-B-C-D-E-F-G, that are the  
15 better substrates for the protease will be cleaved, and can be readily identified because the fluoescor, A, will be cleaved from its nearby quencher F, in a time dependent manner which can be easily quantified. The fluorescent quenching by F of A only occurs when the two are in nearby  
20 proximity, normally within 30 angstrom units. Hence cleavage of the bond D-E allows F to move further away from A and thus allow A to fluoresce when excited by light of the correct wavelength.

1. In this manner the most active compound can be  
25 rapidly identified without the need for further resynthesis and deconvolution. Moreover, the wells

the most efficient substrate can be found by its disappearance into its two component parts, e.g A-B-C-D and E-F-G.

Hence the problem of library deconvolution can be overcome  
5 and the most active substrate for the enzyme can be rapidly identified.

In addition, after the initial treatment of the proteolytic enzyme with the library mixtures, L1 and L2, the residual enzymatic activity in each well can be  
10 quantified by the addition of the most potent fluorogenic substrate for the enzyme, S1, which is found in the 16xn compound library. Because of the nature of the library design this can be quickly prepared and purified. If there  
15 is no appearance of increased fluorescence with the known substrate, S1, then the presence of an enzyme inhibitor can be inferred, which again can be quickly identified without the need for resynthesis.

The general description of the library layout will now be described with reference to figures 1 to 14.

20 For example, when  $n=1$  and the library contains 1600 compounds, in the first column of the first row (A1) (Fig.1) in the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will  
25 be one C component,  $C_1$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ) (Fig. 2). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component,  $C_{10}$ , one



D component,  $D_1$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ). In the tenth column of the eighth row (H10) in the first plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will  
5 be one C component,  $C_{10}$ , one D component,  $D_9$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ). Hence all 1600 components are present in the one plate, because the 80 wells each contain 20 components.

A second complementary library is synthesised as follows  
10 (Fig. 3). In the first column of the first row (A1) of the first plate (P1) of the library, L2, (hereinafter designated as location A1,P1,L2), there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_1$ , and one E component,  $E_1$ . In the tenth column of the  
15 first row (A10) of the first plate (P1) of the library, L2, (hereinafter designated as location A10,P1,L2), there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_{10}$ , and one E component,  $E_1$ . In the first column of the second row (B1) of the first plate (P1) of  
20 the library, L2, (hereinafter designated as location B1,P1,L2), there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_1$ , and one E component,  $E_1$ . In the tenth column of the second row (B10) of the first plate (P1) of the library, L2, (B10,P1,L2)  
25 there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_{10}$ , and one E component,  $E_1$ . Hence only the first two rows are used to accommodate 400 compounds in total.

location A1,P2,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>1</sub>, and one E component, E<sub>1</sub> (Fig. 4). In the tenth column of the first row (A10) of the second plate (P2) of the library, L2, (hereinafter designated as location A10,P2,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>10</sub>, and one E component, E<sub>1</sub>. In the first column of the second row (B1) of the second plate (P2) of the library, L2, (hereinafter designated as location B1,P2,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>1</sub>, and one E component, E<sub>2</sub>. In the tenth column of the second row (B10) of the second plate (P2) of the library, L2, (B10,P2,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>10</sub>, and one E component, E<sub>2</sub>. Hence only the first two rows are used to accommodate 400 compounds in total.

In the first column of the first row (A1) of the third plate (P3) of the library, L2, (hereinafter designated as location A1,P3,L2), there will be ten C components, two D components (D<sub>5</sub> and D<sub>6</sub>), one B component, B<sub>1</sub>, and one E component, E<sub>1</sub> (Fig 5). In the tenth column of the first row (A10) of the third plate (P3) of the library, L2, (hereinafter designated as location A10,P3,L2), there will be ten C components, two D components (D<sub>5</sub> and D<sub>6</sub>), one B component, B<sub>10</sub>, and one E component, E<sub>1</sub>. In the first column of the second row (B1) of the third plate (P3) of the library, L2, (hereinafter designated as location B1,P3,L2), there will be ten C components, two D components (D<sub>5</sub> and D<sub>6</sub>), one B component, B<sub>1</sub>, and one E component, E<sub>2</sub>. In the tenth column of the second row (B10)

of the third plate (P3) of the library, L2, (B10,P3,L2), there will be ten C components, two D components ( $D_7$  and  $D_8$ ), one B component,  $B_{10}$ , and one E component,  $E_1$ . Hence only the first two rows are used to accommodate 400 compounds in total.

In the first column of the first row (A1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A1,P4,L2), there will be ten C components, two D components ( $D_7$  and  $D_8$ ), one B component,  $B_1$ , and one E component,  $E_1$  (Fig. 6). In the tenth column of the first row (A10) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A10,P4,L2), there will be ten C components, two D components ( $D_7$  and  $D_8$ ), one B component,  $B_{10}$ , and one E component,  $E_1$ . In the first column of the second row (B1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location B1,P4,L2), there will be ten C components, two D components ( $D_7$  and  $D_8$ ), one B component,  $B_1$ , and one E component,  $E_2$ . In the tenth column of the second row (B10) of the fourth plate (P4) of the library, L2, (B10,P4,L2), there will be ten C components, two D components ( $D_7$  and  $D_8$ ), one B component,  $B_{10}$ , and one E component,  $E_2$ . Hence only the first two rows are used to accommodate 400 compounds in total.

In this fashion two complementary libraries, L1 and L2 are prepared. In library, L1, each of the 80 of wells contains a mixture of 20 components providing 1600

and furnishing the same 1600 compounds in library, L1, but in a format in which no two compounds found together in library, L1, will be found together in library, L2.

Thus it is an important part of the invention that the compounds contained in the two libraries L1 and L2 are themselves complementary, in that any two compounds which are found together in a 20 component mixture in the same location (e.g. A1P1L1) in library L1, are not found together in any of the 20 component mixtures in any location of the library L2.

In analogous examples, where separately  $n=2, 3$  or  $4$ , extra plates are constructed in library, L1, format to accommodate the component pairs  $E_3$  and  $E_4$  ( $n=2$ ),  $E_5$  and  $E_6$  ( $n=3$ ), and  $E_7$  and  $E_8$  ( $n=4$ ), respectively. For the respective deconvolution libraries of the type, L2, the respective rows in the plates P1, P2, P3, and P4, are increasingly filled with the paired components  $D_1$  and  $D_2$ ,  $D_3$  and  $D_4$ , and  $D_5$  and  $D_6$ , and  $D_7$  and  $D_8$ , respectively.

For example, when  $n=3$ , and the library contains 4800 compounds, in the first column of the first row (A1) in the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will be one C component,  $C_1$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component,  $C_{10}$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ). In the tenth column of the eighth row (H10) in the first

plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component,  $C_1$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ). Hence 1600 components are present  
5 in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the second plate (P2) of the library L1, (hereinafter designated as location A1,P2,L1) there will be one C component,  $C_1$ , one  
10 D component,  $D_1$ , the ten B components and the two E components ( $E_3$  and  $E_4$ ). In the tenth column of the first row (A10) in the second plate (P2) of the library L1, (hereinafter designated as location A10,P2,L1) there will be one C component,  $C_{10}$ , one D component,  $D_1$ , the ten B  
15 components and the two E components ( $E_3$  and  $E_4$ ). In the tenth column of the eighth row (H10) in the second plate (P2) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component,  $C_{10}$ , one D component,  $D_8$ , the ten B components and the two E  
20 components ( $E_3$  and  $E_4$ ). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the third plate (P3) of the library L1, (hereinafter designated as location A1,P3,L1) there will be one C component,  $C_1$ , one  
25 D component,  $D_1$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ). In the tenth column of the first

row (A10) in the third plate (P3) of the library L1, (hereinafter designated as location A10,P3,L1) there will be one C component,  $C_{10}$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_3$  and  $E_4$ ). In the tenth column of the eighth row (H10) in the third plate (P3) of the library L1, (hereinafter designated as location H10,P3,L1) there will be one C component,  $C_{10}$ , one D component,  $D_8$ , the ten B components and the two E components ( $E_3$  and  $E_4$ ). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components.

components and the two E components ( $E_3$  and  $E_4$ ). In the tenth column of the eighth row (H10) in the third plate (P3) of the library L1, (hereinafter designated as location H10,P3,L1) there will be one C component,  $C_{10}$ , one C component,  $C_9$ , the ten B components and the two E components ( $E_3$  and  $E_4$ ). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components. In total the three plate, P1, P2 and P3, contain 1600 compounds/plate 4800 compounds in total.

10 For example, when  $n = 4$ , and the library contains 6400 compounds, in the first column of the first row (A1) in the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will be one C component,  $C_1$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ) (Fig. 7). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component,  $C_{10}$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ). In the tenth column of the eighth row (H10) in the first plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component,  $C_{10}$ , one D component,  $D_8$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ). Hence all 1600 components are present in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the second plate (P2) of the library L1, (hereinafter designated as location A1,P2,L1) there will be one C component,  $C_1$ , one D component,  $D_1$ , the ten B components and the two E

30

components ( $E_3$  and  $E_4$ ) (Fig. 8). In the tenth column of the first row (A10) in the second plate (P2) of the library L1, (hereinafter designated as location A10,P2,L1) there will be one C component,  $C_{10}$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_3$  and  $E_4$ ). In the tenth column of the eighth row (H10) in the second plate (P2) of the library L1, (hereinafter designated as location H10,P2,L1) there will be one C component,  $C_{10}$ , one D component,  $D_8$ , the ten B components and the two E components ( $E_3$  and  $E_4$ ).

In the first column of the first row (A1) in the third plate (P3) of the library L1, (hereinafter designated as location A1,P3,L1) there will be one C component,  $C_1$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_5$  and  $E_6$ ) (Fig. 9). In the tenth column of the first row (A10) in the third plate (P3) of the library L1, (hereinafter designated as location A10,P3,L1) there will be one C component,  $C_{10}$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_5$  and  $E_6$ ). In the tenth column of the eighth row (H10) in the third plate (P3) of the library L1, (hereinafter designated as location H10,P3,L1) there will be one C component,  $C_{10}$ , one D component,  $D_8$ , the ten B components and the two E components ( $E_5$  and  $E_6$ ).

In the first column of the first row (A1) in the fourth plate (P4) of the library L1, (hereinafter designated as location A1,P4,L1) there will be one C component,  $C_1$ , one

the library L1, (hereinafter designated as location A10,P4,L1) there will be one C component,  $C_{11}$ , one D component,  $D_1$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ). In the tenth column of the eighth row (H10) in the fourth plate (P4) of the library L1, (hereinafter designated as location H10,P4,L1) there will be one C component,  $C_{10}$ , one D component,  $D_2$ , the ten B components and the two E components ( $E_1$  and  $E_2$ ).

A second complementary library is synthesised as follows. In the first column of the first row (A1) of the first plate (P1) of the library, L2, (hereinafter designated as location A1,P1,L2), there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_1$ , and one E component,  $E_1$  (Fig. 11). In the tenth column of the first row (A10) of the first plate (P1) of the library, L2, (hereinafter designated as location A10,P1,L2), there will be the ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_{10}$ , and one E component,  $E_1$ . In the first column of the eighth row (H1) of the first plate (P1) of the library, L2, (hereinafter designated as location H1,P1,L2), there will be the ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_1$ , and one E component,  $E_2$ . In the tenth column of the eighth row (H10) of the first plate (P1) of the library, L2, (H10,P1,L2) there will be the ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_{10}$ , and one E component,  $E_2$ . Hence the matrix containing all ten columns and all eight rows are used to accommodate 1600 compounds in total.

In the first column of the first row (A1) of the second plate (P2) of the library, L2, (hereinafter designated as



location A1,P2,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>1</sub>, and one E component, E<sub>1</sub> (Fig. 12). In the tenth column of the first row (A10) of the second plate (P2) of the library, L2,  
5 (hereinafter designated as location A10,P2,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>10</sub>, and one E component, E<sub>1</sub>. In the first column of the second row (B1) of the second plate (P2) of the library, L2, (hereinafter designated as location  
10 B1,P2,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>1</sub>, and one E component, E<sub>2</sub>. In the tenth column of the eighth row (H10) of the second plate (P2) of the library, L2, (H10,P2,L2), there will be ten C components, two D components (D<sub>3</sub> and  
15 D<sub>4</sub>), one B component, B<sub>10</sub>, and one E component, E<sub>8</sub>.

In the first column of the first row (A1) of the third plate (P3) of the library, L2, (hereinafter designated as location A1,P3,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>1</sub>, and one E  
20 component, E<sub>1</sub> (Fig. 13). In the tenth column of the first row (A10) of the third plate (P3) of the library, L2, (hereinafter designated as location A10,P3,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>10</sub>, and one E component, E<sub>1</sub>. In the first  
25 column of the second row (B1) of the third plate (P3) of the library, L2, (hereinafter designated as location B1,P3,L2), there will be ten C components, two D components (D<sub>3</sub> and D<sub>4</sub>), one B component, B<sub>1</sub>, and one E

there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_{10}$ , and one E component,  $E_1$ .

In the first column of the first row ( $A_1$ ) of the fourth plate ( $P_4$ ) of the library,  $L_2$ , (hereinafter designated as location  $A_1, P_4, L_2$ ), there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_1$ , and one E component,  $E_1$  (Fig.14). In the tenth column of the first row ( $A_{10}$ ) of the fourth plate ( $P_4$ ) of the library,  $L_2$ , (hereinafter designated as location  $A_{10}, P_4, L_2$ ), there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_{10}$ , and one E component,  $E_1$ . In the first column of the second row ( $B_1$ ) of the fourth plate ( $P_4$ ) of the library,  $L_2$ , (hereinafter designated as location  $B_1, P_4, L_2$ ), there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_1$ , and one E component,  $E_2$ . In the tenth column of the eighth row ( $H_{10}$ ) of the fourth plate ( $P_4$ ) of the library,  $L_2$ , ( $H_{10}, P_4, L_2$ ), there will be ten C components, two D components ( $D_1$  and  $D_2$ ), one B component,  $B_{10}$ , and one E component,  $E_3$ .

The invention will now be described by reference to the following examples.

**Example 1:**

In this Example the proteolytic enzyme of interest is Der P1, which is found in house dust mite faeces. The example illustrates the synthesis of a number of FRET compounds in which the suitable bond is an unsubstituted amide bond, their use as a library for screening for potential

substrates of Der P1, and subsequent identification and synthesis of active inhibitors of the enzyme.

#### Purification of Der pI.

Crude mite extract (~100mg, SmithKline-Beecham, U.K) was  
5 dissolved in 5mL Phosphate Buffered Saline (PBS; 50 mM  
potassium phosphate; pH 7.4 containing 150 mM NaCl Der pI  
was purified by affinity column chromatography using 4C1  
antibody (indoor Biotechnology, Deeside, U.K.) The crude  
preparation was mixed with ~2 mL of affinity resin for 2 h  
10 at 4°C and then washed with 2-3 volumes of PBS. Elution of  
bound protein was carried out using 5mM glycine containing  
50%(v/v) ethylene glycol. Fractions(2.2 mL) were collected  
and neutralised with 0.8 mL of 0.2 M sodium phosphate  
buffer, pH 7.0. The fractions were pooled and dialysed  
15 overnight against 4 L PBS followed by a second dialysis  
against 2 L PBS for 2-3h. The total protein was  
concentrated as required by ultrafiltration (MacroSep;  
Flowgen, U.K.)

#### Synthesis of compounds

2 The compounds were synthesised using the Multipin  
approach: using Fmoc-Rink amide Macro crowns (Chiron  
Mimotypes Pty., Ltd., 11 Duerdin Street, Clayton Victoria  
3168, Australia) with a loading of 7  $\mu$ Moles.

The amino acid residues of each of the compounds were

acids and activated pentafluorophenyl esters, in which the side-chains are protected using acid labile protecting groups known to those skilled in the art, such as Boc- (for the  $-NH_2$  of Lysine,  $-NH_2$  of anthranilic acid and guanidino of arginine), tBu- (for the  $-OH$  groups of serine, threonine and tyrosine), t-Bu for the  $-COOH$  group of Aspartic acid and Glutamic acid, Trityl- (for the Amide of Asparagine and Glutamine, and the amine functionality of the Histidine ring.

10 The N- $\alpha$ -fluorenylmethoxycarbonyl protecting group of the coupled residues were cleaved using 20% piperidine in dimethylformamide (DMF) for 30 minutes at 20° C. The coupling reactions for the free acids such as Boc-ABz-OH (Boc-2-aminobenzoic acid), and Fmoc-(3-nitro)tyrosine-OH  
15 were accomplished using 10 equivalents of a mixture of the free acid (1 eq.) : TBTU (0.98 eq.) : HOBt (0.98 eq.) : N-methylmorpholine (1.96 eq.) in dimethylformamide (500  $\mu$ L) as solvent for 5 hours at 20° C. The other amino acids were coupled as their pentafluorophenyl esters<sup>26</sup> for 2-6 hours.

20 Hence, in order to couple approximately equal ratios of each component in the mixture of the derivatised amino acids as their pentafluorophenyl esters, a solution of a total of 0.98 equivalents (relative to the amino group loading on the crown) of the mixture of amino acid  
25 pentafluorophenyl esters : HOBt (1 eq.) in DMF (500  $\mu$ L) were coupled for 16 hours at 20° C. The pins were then washed well with DMF and then recoupled using the same mixture under the same conditions. A third coupling of 10  
30 equivalents (relative to the amino group loading of the crown) for 2 hours in DMF was performed using this

coupling protocol with equimolar mixtures of the derivatised pentafluorophenyl esters of the amino acids in slightly less than 1 equivalent, it is possible to obtain approximately equal amounts of the coupled products to the crown. In this fashion the libraries are constructed with 20 compounds present on each crown. The compounds were cleaved from the crowns directly into the 80 designated wells of the desired 96 well plate. In the cleavage protocol each crown was treated with a mixture (600  $\mu$ L) containing trifluoroacetic acid (95%), triethylsilane (5%) for 2 hours at 20° C. The crowns were then washed with trifluoroacetic acid (500  $\mu$ L) and this was then combined with the cleavage solution.

The Fmoc-Rink amide Macro crowns (Chiron Mimotypes Pty., Ltd., 11 Duerdin Street, Clayton Victoria 3168, Australia) at 7  $\mu$ Mol loading per crown, were coupled with a 10 fold excess of a mixture containing L-Fmoc-Asp(O-t-Bu)-OH (1eq) using TBTU (0.98 eq) and N-methylmorpholine (1.96 eq.) in the presence of HOBt (0.98 eq.) in DMF at 0.14M concentration. After deblocking of the Fmoc group with 20% piperidine in DMF for 30 minutes and subsequent washing with DMF and then methanol, coupling of the Fmoc-(3-nitro)tyrosine-OH was accomplished using 10 equivalents of a mixture of the Fmoc-(3-nitro)tyrosine-OH (1 eq.) :TBTU (0.98 eq.): HOBt (0.98 eq.) : N-methylmorpholine (1.96 eq.) in dimethylformamide as solvent at 0.14 M concentration for 5 hours at 20° C. Removal of the Fmoc group (vide infra) was followed by coupling of the

In a particular example the amino acids comprising group B include Ala, Val, Leu, Ser, Asn, Gln, Glu, Lys, Phe, Pro. The amino acids comprising group C include Ala, Val, Leu, Ser, Asn, Gln, Glu, Lys, Phe, Pro. The amino acids  
5 comprising group D include Ala, Val, Ile, Leu, Nle, Ser, Glu, Phe. For  $n=4$ , the amino acids comprising group E include Ala, Val, Ile, Leu, Nle, Ser, Glu, Phe. Otherwise any selection from the amino acids can be made for  $n=1, 2$ , or 3.

10 The plates containing the combined cleavage solutions and were then evaporated to dryness to yield the component mixtures using a rotary centrifuge ("SPEEDVAC", Savant Instruments Inc., Farmingdale, NY) at 800 rpm for 1 hour at  $20^{\circ}$  C under a reduced pressure of  $10^{-2}$  mmHg. Each  
15 component was then transferred to the final mother plate using a (50%: 45%: 5%) mixture of acetonitrile: water: acetic acid. The plates were then lyophilised to dryness using at  $20^{\circ}$  C under a reduced pressure of  $10^{-2}$  mmHg, and then stored at  $-20^{\circ}$  C. In this fashion libraries of the  
20 type shown in Figures 2-14 were prepared.

In further detail, the Multipin approach which was employed is described below:

#### **Multipin Synthesis Of Potential Substrates of Der pI**

The 'Chiron' multipin kit consists of a standard 8 x 12  
25 pin holder containing 96 'pin stems' to which are reversibly attached 'crowns'. The 'crowns' provide a reactive polymer surface upon which a growing peptide is anchored during solid phase peptide synthesis. Each crown

(the equivalent of the peptide-resin in standard solid phase synthesis) can be considered to be an independent reactor by performing simultaneous synthesis in individual 1mL wells of industry standard 96 well plates. Each well, and thus each crown, can be charged with a unique set of reagents providing unique sequences to each crown. Common steps such as washing or removal of N $\alpha$  protection can be performed concomitantly.

Synthesis is based upon the use of N $\alpha$ -fluorenylmethyloxycarbonyl (Fmoc) protected amino acids. Side-chains of tri-functional amino acids are protected with acid labile groups such as trityl or tert-butyl. The addition of amino-acid residues to the growing peptide chain, a process termed 'coupling' proceeds through the utilisation of pre-formed pentafluorophenyl (pfp) esters or activation of the free acid, using the reagents HBTU or BOP in the presence of tertiary base (NMM) and HOBT as catalyst.

The experimental techniques used are fully documented (Maeji, N. J. Bray, A. M. Valerio, R. M. and Wang, W., *Peptide Research*, 8(1), 33-38, 1995 and Valerio, R. M. Bray, A. M. and Maeji, N. J. *Int. J. Pept. Prot. Res.* 44, 158-165, 1994 and the main steps are briefly as follows.

#### General Methods

##### Preparation of Multipin Assembly

onto stems and slotted into the 8 x 12 stem holder in the desired pattern for synthesis.

#### Removal of N $\alpha$ -Fmoc Protection

A 250mL solvent resistant bath is charged with 200 ml of a  
5 20% piperidine/DMF solution. The multipin assembly is added and deprotection allowed to proceed for 30 minutes. The assembly is then removed and excess solvent removed by brief shaking. The assembly is then washed consecutively with (200mL each), DMF (5mins) and MeOH (5mins, 2mins,  
10 2mins) and left to air dry for 15mins.

#### Quantitative UV Measurement of Fmoc Chromophore Release

A 1cm path length UV cell is charged with 1.2mL of a 20% piperidine/DMF solution and used to zero the absorbance of the UV spectrometer at a wavelength of 290nm. A UV  
15 standard is then prepared consisting of 5.0mg Fmoc-Asp(OBut)-Pepsyn KA (0.08mmol/g) in 3.2mL of a 20% piperidine/DMF solution. This standard gives  $Abs_{290} = 0.55-0.65$  (at RT). An aliquot of the multipin deprotection solution is then diluted as appropriate to give a  
20 theoretical  $Abs_{290} = 0.6$ , and this value compared with the actual experimentally measured absorbance showing the efficiency of previous coupling reaction.

#### Coupling of amino-acid residues

Whilst the multipin assembly is drying, the appropriate  
25 N $\alpha$ -Fmoc amino acid pfp esters (10 equivalents calculated from the loading of each crown) and HOBt (10 equivalents)



required for the particular round of coupling are accurately weighed into suitable containers. Alternatively, the appropriate N $\alpha$ -Fmoc amino acids (10 equivalents calculated from the loading of each crown),  
5 desired coupling agent e.g. HBTU (9.9 equivalents calculated from the loading of each crown) and activation eg HOBt (9.9 equivalents calculated from the loading of each crown), NMM (19.9 equivalents calculated from the loading of each crown) are accurately weighed into  
10 suitable containers.

The protected and activated Fmoc amino acid derivatives are then dissolved in DMF (500 $\mu$ l for each macrocrown, e.g. for 20 macrocrowns, 20 x 10eq x 7mmoles of derivative would be dissolved in 10 000 $\mu$ L DMF). The appropriate  
15 derivatives are then dispensed to the appropriate wells ready for commencement of the 'coupling cycle'. As a standard, coupling reactions are allowed to proceed for 2-6 hours (depending upon nature of coupling e.g. Ala to Ala 2 hours Val to Leu 6 hours).

20 When coupling Fmoc amino-acid pentafluorophenyl esters, 10eq of derivative in DMF (400 $\mu$ l) with bromophenol blue stock solution (100 $\mu$ l) is used for each macrocrown. This allows monitoring of the progress of the acylation reaction through the disappearance of the deep blue  
25 coloration of bromophenol blue in the presence of unreacted amine to a pale yellow upon completion of acylation.

Bromophenol blue (20mg) is dissolved in DMF (50mL) and HOBt (10mg) added.

#### Washing Following Coupling

If a 20% piperidine/DMF deprotection is to immediately follow the coupling cycle, then the multipin assembly is briefly shaken to remove excess solvent washed consecutively with (200mL each), MeOH (5mins) and DMF (5mins) and deprotected (see above). If the multipin assembly is to be stored, then a full washing cycle consisting brief shaking then consecutive washes with (200mL each), DMF (5mins) and MeOH (5mins, 2mins, 2mins) is performed.

#### Acidolytic Mediated Cleavage of Peptide-Pin Assembly

Acid mediated cleavage protocols are strictly performed in a fume hood. A polystyrene 96 well plate (1mL / well) is labelled, then the tare weight measured to the nearest mg. Appropriate wells are then charged with a trifluoroacetic acid / triethylsilane (95:5, v/v, 600 $\mu$ l) cleavage solution, in a pattern corresponding to that of the multipin assembly to be cleaved.

The multipin assembly is added, the entire construct covered in tin foil and left for 2hrs. The multipin assembly is then added to another polystyrene 96 well plate (1mL /well) containing trifluoroacetic acid / triethylsilane (95:5, v/v, 600 $\mu$ l) (as above) for 5 mins.

The cleaved assembly is washed with DMF (200 $\mu$ L, 5mins), MeOH (200 $\mu$ L, 5mins), the spent crowns removed and discarded, the stems removed and washed by sonication in methanol (1hr, RT).

5      **Work up of Cleaved Peptides**

The primary polystyrene cleavage plate (2hr cleavage) and the secondary polystyrene plate (5min wash) (see above) are then placed in the SpeedVac and the solvents removed (minimum drying rate) for 90mins.

10     The contents of the secondary polystyrene plate (see above) are transferred to their corresponding wells on the primary plate using an acetonitrile / water / acetic acid (50:45:5, v/v/v) solution (3 x 150 $\mu$ l) and the spent secondary plate discarded.

15     **Analysis of Products**

1.0 $\mu$ l of each well (see above) is diluted to 400 $\mu$ l with 0.1%aq TFA and analysed by HPLC-MS. Column Vydac C4 (214TP52, narrowbore, 21 x 250mm). Eluents :- Solvent A = 0.1%aq trifluoroacetic acid, Solvent B = acetonitrile / 10%A. Gradient :- 10-90% B in A over 27mins, 250ml / min, 215nm UV detection. The individual substrates described below were prepared by the above methods and shown by HPLC-MS to be >95% with the correct mass.

The primary polystyrene plate (plus the washings from the secondary plate) is covered with tin foil, held to the plate with an elastic band. A pin prick is placed in the foil directly above each well and the plate placed at  
5 -30°C for 30mins. The plate is then lyophilised on the 'Heto freeze drier' overnight. Where appropriate individual peptides were then weighed and dissolved to 10mM stock solutions in DMSO prior to biological screening. Alternatively the 20 component mixture is  
10 weighed and the peptide/20 component ratio is calculated.

Further coupling of amino acid residues was carried out according to the multipin approach described above. Whilst the multipin assembly was drying, the appropriate N $\alpha$ -Fmoc amino acids (10 equivalents calculated from the loading of  
15 each crown), HATU coupling agent (9.9 equivalents calculated from the loading of each crown), HOAt catalyst (9.9 equivalents calculated from the loading of each crown) and DIPEA (19.9 equivalents calculated from the loading of each crown) were accurately weighed into  
20 suitable containers.

The protected N $\alpha$ -Fmoc amino acids and coupling agents were then dissolved in DMF (500  $\mu$ l for each macrocrown) and activated by the addition of DIPEA. The appropriate derivatives were then dispensed to their appropriate wells  
25 and as standard coupling to each macro crown was allowed to proceed for 2 hours.

When coupling particularly hindered amino acid residues such as N-Methyl, C $\alpha$ -Methyl or unusual amino acids (whose

coupling efficiency is unknown) the coupling reaction was repeated, as standard, for a further 2 hours.

### Substrates for *Der pI*

Using the general techniques described above, the following compounds were prepared and assayed as potential substrates against *Der pI* purified as described above.

	Peptide	Measured $K_m$ ( $\mu M$ )
	Abz-Val-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	12
	H-Val-Ala-Nle-Ser-TyrNO <sub>2</sub> -Asp-NH <sub>2</sub>	NS
10	H-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Ac-Val-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	H-Val-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-NH <sub>2</sub>	NS
	H-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Ac-Val-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-NH <sub>2</sub>	NS
15	Abz-Val-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-NH <sub>2</sub>	NM
	Abz-Val-Ala-Nle-Ser-NH <sub>2</sub>	NM
	Abz-Val-Ala-Nle-Ser-Phe-Asp-NH <sub>2</sub>	NM
	Abz-Val-Ala-Nle-Ser-Tyr-Asp-NH <sub>2</sub>	NM
	Abz-Val-Ala-Nle-Ser-Ala-Asp-NH <sub>2</sub>	NM
20	Abz-Val-Ala-Nle-Ser-Lys-Asp-NH <sub>2</sub>	NM
	Abz-Val-Ala-Nle-Ser-eAHA-Asp-NH <sub>2</sub>	NM
	Abz-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NM
	Abz-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Bz-Val-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-NH <sub>2</sub>	NM <sup>a</sup>
25	Bz-(2-carboxy)-Val-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-NH <sub>2</sub>	NM <sup>a</sup>

<sup>a</sup> NS = No substrate; NM = No measurement.

	Bz-Val-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-NH <sub>2</sub>	NM <sup>1</sup>
	Abz-Val-Ala-Lys-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	14
	Abz-Val-Ala-Gln-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	6
	Abz-Val-Ala-Thr-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	6
5	Abz-Val-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	4
	Abz-Val-Ala-Cha-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	5
	Abz-Val-Ala-His-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	> 20
	Abz-Val-Ala-ACH-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-Ala-DNle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
10	Abz-Val-Ala-3pyr-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	10
	Abz-Val-Ala-Hyp-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-Ala-ACP-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-Lys-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	35
	Abz-Val-DAla-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
15	Abz-Val-Tic-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-ACH-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-Met(O)-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	35
	Abz-Val-2Nal-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-ACP-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
20	Abz-Val-DLys-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-DGln-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-3pyr-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-Cha-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-DVal-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
25	Abz-Gln-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	12
	Abz-Lys-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	>15
	Abz-Tic-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-ACH-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Met(O)-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	20
30	Abz-3pyr-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	>10
	Abz-2Nal-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	15
	Abz-Leu-Ala-hLeu-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	18

	Abz-Cha-Ala-hLeu-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	9
	Abz-Bip-Ala-hLeu-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	2.5
	Abz-Bip-Ala-hLeu-Tyr-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	3
	Abz-Bip-Ala-hLeu-Leu-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	3.7
5	Abz-Bip-Ala-hLeu-Lys-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	2
	Abz-Bip-Ala-hLeu-Asp-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	5.0
	Abz-Bip-Ala-hLeu-Abu-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	1.7
	Abz-Bip-Ala-hLeu-Cha-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	2.5
	Abz-Bip-Ala-hLeu-Met (O) -Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	5
10	Abz-Bip-Ala-hLeu-Thr-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	2.5
	Abz-Bip-Ala-hLeu-3pyr-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	4
	Abz-Bip-Ala-hLeu-Bu <sup>t</sup> Gly-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	4
	Abz-Bip-Ala-hLeu-Hyp-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	4
	Abz-Phe-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	NM
15	Abz-3.Pyr-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	NM
	Abz-1.Naph-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	17
	Abz-2.Naph-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	NM
	Abz-Tyr-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	NM
	Abz-Bip-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	10
20	Abz-Lys-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	15
	Abz-Glu-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	20
	Abz-Leu-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	NM
	Abz-Hyp-Val-Ala-Nle-Ser-Tyr (NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	NS

NS indicates that the peptide was not hydrolysed by Der  
 25 pI.

NM indicates that the peptide was a substrate for Der pI,  
 but the K<sub>m</sub> was not measured.

NM<sup>1</sup> indicates that the peptide was a substrate for Der pI<sup>1</sup>.

Ranked in order of cleavage rate :-Bz > n-But > Piv >  
Bz(2-carboxy) > Abz.

Coupling of amino acid residues was carried out according  
to the multipin approach described above. Whilst the  
5 multipin assembly was drying, the appropriate N $\alpha$ -Fmoc  
amino acids (10 equivalents calculated from the loading of  
each crown), HATU coupling agent (9.9 equivalents  
calculated from the loading of each crown), HOAt catalyst  
(9.9 equivalents calculated from the loading of each  
10 crown) and DIPEA (19.9 equivalents calculated from the  
loading of each crown) were accurately weighed into  
suitable containers.

The protected N $\alpha$ -Fmoc amino acids and coupling agents were  
then dissolved in DMF (500  $\mu$ l for each macrocrown) and  
15 activated by the addition of DIPEA. The appropriate  
derivatives were then dispensed to their appropriate wells  
and standard coupling to each macrocrown was allowed to  
proceed for 2 hours.

When coupling particularly hindered amino acid residues  
20 such as N-Methyl, C $\alpha$ -Methyl or unusual amino acids (whose  
coupling efficiency is unknown) the coupling reaction was  
repeated, as standard, for a further 2 hours.

The following sequences were synthesised in this way:

	Peptide Sequence	Measured Km( $\mu$ M)
25	Abz-Val-Ala- (NMe)Nle-Ser-Tyr(NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	NS
	Abz-Val- (NMe)Ala-Nle-Ser-Tyr(NO <sub>2</sub> ) -Asp-NH <sub>2</sub>	NS



	Abz-Val-Ala-Aib-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Val-Aib-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Deg-Ala-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	nBu-D.Ser-D.Nle-D.Ala-D.Val-p.Aba-NH <sub>2</sub>	NS
5	Bz-Val-Ala-Statine-Ser-eAha-NH <sub>2</sub>	NS
	Abz-p.Aba-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS
	Abz-Cmpi-Nle-Ser-Tyr(NO <sub>2</sub> )-Asp-NH <sub>2</sub>	NS

NS-Not Substrate: Not hydrolysed by *Der pI*

10 NM-Substrate but not measured: Substrate for *Der pI* but not measured.

#### Assay procedure

Each mixture of 20 compounds in the libraries of the apparatus described herein was screened at a concentration of 1.0  $\mu$ M per compound in an assay against the cysteinyl  
 15 protease *Der pI*. The most active wells were identified by the rate of emission of fluorescence at 420 nm when the samples were irradiated at 320 nm. An analysis of the two complementary libraries showed that the best substrates for the enzyme were:

16 Abz-B-C-D-E-Tyr(NO<sub>2</sub>)-Asp-NH<sub>2</sub>

Where

B= Valine>Alanine, Glutamine, Leucine, Phenylalanine

C= Alanine>>Glutamine, or Lysine.

D= Leucine, Norleucine or Alanine>Serine

20 E=Serine

Abz-Val-Ala-Nle-Ser-Tyr(NO<sub>2</sub>)-Asp-NH<sub>2</sub>

This compound was then resynthesised as a single component using the peptide synthesis methodology described herein. The  $k_{cat}/K_m$  value for the pure substrate in the *Der* pI assay was measured as  $3.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , and was considered to be suitably high for use in a high throughput assay for the general screening of inhibitors of *Der* pI.

**High throughput assay development**

Plate assays were carried out in 96 well plate format, using 0.1 ug of *Der* pI per 100  $\mu\text{L}$  assay volume in each well and using 20  $\mu\text{M}$  of the substrate. All assays were performed in Assay Buffer (AB; 50 mM potassium phosphate, pH 8.25 containing 1mM ethylenediaminetetraacetic acid (EDTA) and 1mM dithiothreitol (DTT). The *Der* pI enzyme is pre-activated by addition of DTT and this is incubated at room temperature for 5 min. prior to initiation of the assay. As an example for the screening methodology, each well contains a 5  $\mu\text{L}$  of a 20  $\mu\text{M}$  solution of the test compound in DMSO, 10  $\mu\text{L}$  of a 200  $\mu\text{M}$  aqueous solution of the substrate, 2, and 85  $\mu\text{L}$  of *Der* pI in AB is added to initiate the reaction. Enzyme activity is monitored by fluorescence using 320 nm for excitation and 420nm for the emission wavelengths using a Labsystems Fluroskan Ascent machine. Kinetic measurements were carried out using a Hitachi F-4500 Fluorescence Spectrophotometer.

**Synthesis of inhibitors of *Der* pI**

The best substrate described above was shown by HPLC-mass spectroscopic analysis of the enzyme/ assay solution, to be cleaved between the Norleucine-Serine amide bond. Replacement of the terminal Abz group by a series of derivatives (e.g. Boc-, Pivaloyl, Benzoyl, and 2- carboxy-  
5 Benzoyl) affected substrate activity and specificity for the Der pI enzyme. With this knowledge of the P<sub>1</sub>-P<sub>1</sub>' cleavage site and for the P<sub>4</sub>-P<sub>3</sub>-P<sub>2</sub>-P<sub>1</sub> motif, the compound Boc-Val-Ala-Leu-H, 4, was synthesised as shown in Scheme  
10 1a, figure 15.

Attachment of a suitable Michael acceptor such as CH=CH-CO<sub>2</sub>Et, and -CH=CH-SO<sub>2</sub> Ph to the motif (Scheme 2, figure  
15 16), provided active inhibitors of the enzyme with apparent IC<sub>50</sub> values of 50nM, 1000 nM and 100nM respectively.

#### **Preparation of an Acyloxymethylketone Series**

A series of acylomethylketone compounds was prepared by the following procedures:

##### **N-Benzoyl-L-valyl-L-alanyl-L-norleucine**

11 N-Benzoyl-L-valyl-L-alanyl-L-norleucine was prepared by solid phase benzoylated peptide synthesis as follows:

Resin loading (step 1)

minutes. A solution of diisopropylethylamine in DCM(10ml, 57mmol in 30ml) was added over 5minutes and the resulting mixture stirred at room temperature for 2 hours. Methanol (5ml) added and reaction mixture stirred for a further 10minutes before resin filtered and washed with 3x DCM, 2x DMF, 2x 2-propanol, 2x DMF, 2x 2-propanol, methanol, 2x ether and dried under vacuum for 24 hours.

#### Amino acid deprotection (step 2)

Fmoc-L-norleucine loaded resin was deprotected by treatment with 20% piperidine in DMF over 4 hours. The swollen resin was filtered, washed with 5x DMF, 2x ether and dried under vacuum for 24 hours.

#### Peptide chain extension (step 3)

L-Norleucine loaded resin (5mmol) was added to a solution of Fmoc-L-alanine (6.23g, 20mmol), hydroxybenzotriazole (3.0g, 20mmol), 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (7.59g, 20mmol) and diisopropylethylamine (6.97ml, 40mmol) in DMF (20ml) and allowed to swell over 4 hours with mild agitation. Resin was filtered and washed with 4x DMF, 2x ether and dried under vacuum overnight.

Steps (2) and (3) were carried out repetitively with Fmoc-L-alanine and Fmoc-L-valine to afford resin bound tripeptide H-L-valyl-L-alanyl-L-norleucine.

#### Peptide chain benzoylation (step 4)

L-Valyl-L-alanyl-L-norleucine loaded resin (1g, approx. 1mmol) was added to a solution of benzoic acid (0.488g, 4mmol), hydroxybenzotriazole (0.6g, 4mmol), 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium  
5 hexafluorophosphate (1.52g, 4mmol) and diisopropylethylamine (1.40ml, 8mmol) in DMF (5ml) and allowed to swell over 6 hours with mild agitation. Resin was filtered and washed with 4x DMF, 2x ether and dried under vacuum overnight.

10 Resin cleavage (step 5)

N-Benzoyl-L-valyl-L-alanyl-L-norleucine loaded resin (1.0g, approx. 1mmol) was treated with a 1% solution of trifluoroacetic acid in dichloromethane (20ml) containing triethylsilane (320ml, 2mmol) for 1 hour. Resin was  
15 removed by filtration and washed with dichloromethane (3x10ml). Organic layer was collected, evaporated and titrated with ether to afford N-benzoyl-L-valyl-L-alanyl-L-norleucine (285mg). Electrospray-MS m/z 407 [MH<sup>+</sup>].

Bromomethylketone formation (step 6)

20 **N-Benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone**  
N-Benzoyl-L-valyl-L-alanyl-L-norleucine (140mg, 0.34mmol) was suspended in dry THF (3ml) and dry DMF was added dropwise to afford homogeneity. The reaction mixture was cooled to -10°C and isobutylchloroformate (129ml, 1.0mmol)  
25 and N-methylmorpholine (109ml, 1.0mmol) added with stirring

room temperature over 1 hour before a 1:1 solution of acetic acid and 50% HBr (1ml, 3.0mmol HBr) was added dropwise and stirred for 15 minutes. The organic phase was diluted with ethylacetate (40ml), washed with water (10ml), brine (10ml) and sodium bicarbonate (2x10ml), dried over MgSO<sub>4</sub>, solvent removed under vacuum. This afforded an off white solid (152mg) which could be further purified as required by prep. HPLC. Electrospray-MS m/z 482 [MH<sup>+</sup>] and 484 [MH<sup>+</sup>].

10 Acyloxymethylketone formation (step 7)

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2, 6-bis(trifluoromethyl) benzoyloxymethyl ketone**

A mixture of potassium fluoride (0.1mmol, 6mg) and 2,6-bis(trifluoromethyl)benzoic acid (0.066mmol, 17mg) in dry DMF (500ml) was stirred over molecular sieves at room temperature for 5 minutes. A solution of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone (0.033mmol, 16mg) in dry DMF (500ml) was added and the reaction mixture stirred for 1 hour. The reaction mixture was passed through a short silica plug and washed with 5% methanol in dichloromethane. Solvent was removed under vacuum and the residue purified using prep. HPLC. Freeze drying afforded (6.4mg) as a white lyophilisate. Electrospray-MS m/z 660 [MH<sup>+</sup>].

25 Similarly the following compounds were prepared:

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2, 6-dimethylbenzoyloxymethyl ketone** (Electrospray-MS m/z 552

[MH<sup>+</sup>]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine  
bromomethyl ketone and 2,6-dimethylbenzoic acid.

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2-**

**hydroxybenzoyloxymethyl ketone** (Electrospray-MS m/z 540

5 [MH<sup>+</sup>]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine  
bromomethyl ketone and 2-hydroxybenzoic acid.

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2,6-**

**dichlorobenzoyloxymethyl ketone** (Electrospray-MS m/z 592

[MH<sup>+</sup>] and 594 [MH<sup>+</sup>]) from of N-benzoyl-L-valyl-L-alanyl-L-  
10 norleucine bromomethyl ketone and 2,6-dichlorobenzoic  
acid.

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine benzoyloxymethyl**

**ketone** (Electrospray-MS m/z 524 [MH<sup>+</sup>]) from of N-benzoyl-  
L-valyl-L-alanyl-L-norleucine bromomethyl ketone and

15 benzoic acid.

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2,3,4,5,6-**

**pentafluorobenzoyloxymethyl ketone** (Electrospray-MS m/z  
614 [MH<sup>+</sup>]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine  
bromomethyl ketone and 2,3,4,5,6 pentafluorobenzoic acid.

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine 1,1-**

**dimethylpropyloxymethyl ketone** (Electrospray-MS m/z 504  
[MH<sup>+</sup>]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine  
bromomethyl ketone and 1,1-dimethylpropanoic acid.

ketone (Electrospray-MS m/z 504 [MH<sup>+</sup>]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine

valyl-L-alanyl-L-norleucine bromomethyl ketone and N-benzyloxycarbonyl-D-serine-O-tert-butylether.

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine N(-benzyloxycarbonyl)-D-serineoxy methyl ketone**

5 (Electrospray-MS m/z 641 [MH<sup>+</sup>]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and N-benzyloxycarbonyl-D-serine.

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2-furanoxymethyl ketone** (Electrospray-MS m/z 514 [MH<sup>+</sup>]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2-furan  
10 carboxylic acid.

**N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2,6-dichlorophenylacetoxy methyl ketone** (Electrospray-MS m/z 606 [MH<sup>+</sup>], 608 [MH<sup>+</sup>]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2,6-dichlorophenylacetic  
15 acid.

Standard Prep. HPLC conditions were as follows: C4 preparative HPLC system (Vydac, 22x 250mm) eluting at 10ml per minute a gradient of 5-95% (90% acetonitrile (0.1%  
20 TFA)) over 30 minutes.

The following compounds were prepared by the techniques and procedures described beneath each named compound.

**Preparation of Ethyl-(S)-(E)-3-((tert-butoxycarbonyl amino valyl alanyl) amino-6-methyl-hept-2-enoate**



To a suspension of sodium hydride (46mg, 1.9mmol) in anhydrous THF (4ml) cooled to 0°C was added a solution of triethylphosphonoacetate (420mg, 1.9 mmol) in THF (2 ml) dropwise over 5 minutes and the mixture stirred until gas evolution ceased. The solution was added dropwise to a solution of BocVAL-CHO (600mg, 1.56mmol) in dry THF cooled to -10°C. The reaction mixture was stirred for 1 hour and saturated ammonium chloride (10ml) was added. A white solid precipitated which was removed by filtration and the filtrate was partitioned between ethyl acetate and water. The organic layer was dried with magnesium sulphate and evaporated to give an oil which was crystallised from acetonitrile water to yield the title compound, 640mg, 91%.

MS (EI +ve) required  $(M^+(C_{23}H_{41}N_2O_6)+1)=456$ : found  $(M^++H)=456$ ,  $((M^+-BOC)+1)=356$  (100%).

**Preparation of (S)-(E)-3-((tert-butoxy carbonyl amino valyl alanyl) amino-6-methyl-hept-2-enoic acid**

The ethyl ester (455mg, 1mmol) was dissolved in dioxane (10ml) and water added followed by lithium hydroxide (126mg, 3mmol). The solution was stirred for 3 hours and 1M HCl aq was added until the pH reached neutrality. The dioxane was removed by rotary evaporation and the pH adjusted to 4 with 1M HCl aq. The title compound precipitated, filtered and washed with water to yield 420mg, 98%.

**Preparation of 1,1,1-Trifluoroethyl-(S)-(E)-3-((tert-butoxy carbonyl amino valyl alanyl) amino-6-methyl-hept-2-enoate**

The acid (BocVAL-CO<sub>2</sub>H) (50mg, 0.117mmol) and  
5 dimethylaminopyridine (29mg, 0.24mmol) was dissolved in  
dry dichloromethane (1ml) and cooled to 0°C. Water soluble  
carbodiimide hydrochloride salt (26mg, 0.13mmol) in 0.5ml  
dichloromethane was added and the solution stirred for 5  
minutes. 1,1,1-Trifluoroethanol (0.017ml, 0.23mmol) in  
10 0.5ml dichloromethane was added and the reaction was  
allowed to warm to room temperature after 1 hour and the  
reaction mixture stirred overnight. The reaction mixture  
was washed 2x2ml 0.5M citric acid solution, 1x2ml water,  
1x2ml saturated sodium bicarbonate solution, 1x2ml water,  
15 dried with magnesium sulphate and evaporated to dryness to  
give the title compound.

MS (EI +ve) required ( $M^+(C_{23}H_{38}N_3O_6F_3)+1$ )=510: found( $M^+H$ )  
=510, ( $(M^+-BOC)+1$ ) =410, ( $(M^+-tBu)+1$ ) =454 (100%).

**Preparation of Ethyl-(S)-(E)-3-(N-benzoyl valyl alanyl)  
20 amino-6-methyl-hept-2-enoate**

The tert-butoxy carbonyl protected ethyl ester (16.6mg,  
0.036mmol) was dissolved in 4.0M HCl in dioxane (2ml)  
stirred at room temperature for 30 minutes and evaporated  
to dryness. The residue was dissolved in DMF (0.5ml) and  
25 N-methylmorpholine (7.36mg, 0.073mmol) added followed by  
benzoyl chloride (5.4mg, 0.038mmol) in DMF 0.5ml. The  
reaction stirred for 2 hours, diluted with 0.1%  
trifluoroacetic acid solution (4ml) and acetonitrile (2ml)

and injected onto a C4 preparative HPLC system (22x250mm) eluting at 10ml per minute, monitoring at 215nm and a gradient of 10-90% system B over 25 minutes and holding at 90% for 15 minutes. System A = 0.1% TFA in water, system B = 90% acetonitrile, 10% system A. The peak eluting at 26-28 minutes was collected and lyophilised to a white solid, yield 4.5mg, 27%.

Analysis by MS (EI +ve) required  $(M^+(C_{15}H_{13}N_3O_5)+1)=460$ :  
found  $(M^++H)$  -460.

#### 10 Preparation of Diethyl Phenylsulfonylmethylphosphonate

The Diethyl Phenyl sulfonylmethylphosphonate was prepared using a method adapted from I. Shahak, and J. Almog. (Synthesis, 145,1970).

The commercially available diethyl  
15 phenylthiomethylphosphonate (1.0 ml, 4.1 mmol) was dissolved in dichloromethane (10 ml). Sulphuric acid (10 ml, 25 %) was added and the mixture cooled on ice. Solid potassium permanganate was then added in three aliquots of 0.5g with stirring. After the additions the reaction  
20 appeared to be complete. Solid sodium metabisulfite was added slowly until the mixture turned colourless. This was then extracted with ethyl acetate (x3) and the combined organic washings washed with saturated sodium bicarbonate solution followed by brine before drying over sodium  
25 sulphate. The volatiles were removed in vacuo. The residue

In this way the desired product, diethyl phenylsulfonylmethylphosphonate (1.0 g, quant) was obtained as a colourless solid.

The product was analysed by mass spectrometry (MS) (MALDI-TOF): required ( $M^+$  ( $C_{11}H_{13}O_5PS$ ) +1) = 292; obtained ( $M^+$ +1) = 292

**Preparation of (S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-1-phenylsulfonyl-5-methyl-1-hexene**

10 Diethyl phenylsulfonylmethylphosphonate (38 mg, 129 mmol) was dissolved in dry THF (10 ml) and then cooled to 0°C under an atmosphere of nitrogen. Sodium hydride (8 mg of 60% dispersion in oil, 200 mmol) was added and the mixture stirred for 15 mins (effervescence). The aldehyde 'Boc-  
15 Val-Ala-Leu-CHO (50 mg, 129 mmol) was then added to the resulting solution and the mixture was stirred for 60 mins. The reaction was quenched by the addition of dilute hydrochloric acid (0.1 M), followed by extraction with ethyl acetate (x3). The separated organic phase was  
20 sequentially washed with saturated sodium bicarbonate solution and brine before drying over sodium sulphate. The volatiles were removed in vacuo. The residue was purified by flash chromatography on silica eluting with ethyl acetate/hexane 4/6. An unidentified by-product was eluted  
25 first (12 mg) followed by the desired product (S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-phenylsulfonyl-5-methyl-1-hexene (22 mg, 32%) as a solid.

MS (electrospray) required ( $M^+$  ( $C_{26}H_{41}O_6N_3S$ ) +1) = 523: found ( $M^+$ +Na) = 546, ( $(M^+ - \text{Boc}) + 1$ ) = 424 (100%).

**Preparation of Diethyl methylsulfonylmethylphosphonate**

The commercially available Diethyl methylthiomethylphosphonate was converted to the title compound using the method of I. Shahak and J. Almog (Synthesis, 171, 1969).

**Preparation of (S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-1-methylsulfonyl-5-methyl-1-hexene**

Diethyl methylsulfonylmethylphosphonate (30 mg, 130 mmol) was dissolved in dry THF (5 ml) and then cooled to 0°C under an atmosphere of nitrogen. Sodium hydride (7 mg of 60% dispersion in oil, 175 mmol) was added and the mixture stirred for 15 mins (effervescence). The aldehyde <sup>1</sup>Boc-Val-Ala-Leu-CHO (50 mg, 129 mmol) was then added to the resulting solution and the mixture then stirred for 60 mins. The reaction was quenched by addition of dilute hydrochloric acid (0.1 M), followed by extraction with ethyl acetate(x3). The separated organic phase was sequentially washed with saturated sodium bicarbonate solution and brine before drying over sodium sulphate. The volatiles were then removed in vacuo. The residue was purified by flash chromatography on silica eluting with ethyl acetate/hexane 8/2. An unidentified by-product was eluted first (4 mg), followed by the desired product (S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-methylsulfonyl-5-methyl-1-hexene (24 mg, 40%) as a solid.

### Preparation of Ethyl diethylphosphorylmethylsulfonate

Prepared in accordance with procedure B described in L. Ghosez et. al. (Tetrahedron, 43, 5125, 1987).

The product was analysed on MS (electrospray) required  
5 (M<sup>+</sup> (C<sub>12</sub>H<sub>21</sub>O<sub>4</sub>PS)+1)=261: Found (M<sup>+</sup>+H)=261, (M<sup>+</sup>+Na)=283.

### Preparation of Diethyl(S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-5-methylhexenylsulfonate.

Ethyl diethylphosphorylmethanesulfonate (36 ml, ~138mmol)  
10 was dissolved in dry THF (5 ml) and then cooled to 0°C under an atmosphere of nitrogen. Sodium hydride (8 mg of 60% dispersion in oil, 200 mmol) was added and the mixture stirred for 15 mins (effervescence). The aldehyde <sup>3</sup>Boc-Val-Ala-Leu-CHO (50 mg, 129 mmol) was added to the  
15 resulting solution and the mixture stirred for 30 mins. The reaction was quenched by addition of dilute hydrochloric acid (0.1 M), followed by extraction with ethyl acetate (x3). The separated organic phase was sequentially washed with sodium bicarbonate solution and  
20 brine before drying over sodium sulphate. The volatiles were then removed in vacuo. The residue was purified by flash chromatography on silica eluting with ethyl acetate/hexane 1/1. The desired product Diethyl(S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-5-  
25 methylhexenylsulfonate, (22 mg, 35%) was obtained as a solid.

MS(electrospray) required  $(M^+(C_{22}H_{41}O-N_3S)+1)=492$ : found  
 $(M^++1)=492$ ,  $((M^+-tBoc)+1)=392$  (100%)

**Example 2:**

**Design of Depsipeptides**

5 Another suitable bond is an ester bond to form a  
depsipeptide. The incorporation of depsipeptide substrates  
aided the identification of substrates for low reactivity  
viral proteases.

Substrates of the general formula

10 
$$n [Abz-B_{1-12}-C_{1-10}-D_{1-8}y[COO]-E_{1-8}-Tyr(NO_2)-Asp-NH_2]$$

were produced.

However, a significant proportion of viral proteases only  
recognise substrate sequences larger than those  
represented by the general structure above. It is well  
15 acknowledged that by the very nature of action of a viral  
protease (function is to cleave immature viral proteins  
into the mature viral package) one automatically receives  
data concerning the natural substrate sites. Thus, the  
general structure above can be extended by introducing  
20 extra fixed amino-acids at appropriate sites. A logical  
extension would be to introduce the known P1-P1' cleavage  
site as the depsipeptide bond, then subsequently introduce

n [Abz-B<sub>1,11</sub>-C<sub>1,11</sub>-D<sub>1,8</sub>-E<sub>1,8</sub>-P1 y[COO]-P1'-Tyr(NO<sub>2</sub>)-Asp-NH<sub>2</sub>]

Furthermore, if these substrates again proved to be too small, one may use the known substrate sequences to introduce additional fixed positions. For instance, with Hepatitis NS3 protease it is known that the natural P6 position is a conserved acidic residue (aspartic or glutamic acid). Thus one could extend the above structure as detailed below.

n [Abz-P6-B<sub>1,11</sub>-C<sub>1,11</sub>-D<sub>1,8</sub>-E<sub>1,8</sub>-P1 y[COO]-P1'-Tyr(NO<sub>2</sub>)-Asp-NH<sub>2</sub>]

The novel methodology described herein greatly facilitates the invention of therapeutically useful proteolytic enzyme inhibitors and is commercially exploitable. This is because the best substrate motif for the proteolytic enzyme can be rapidly identified, and, since there exist in the literature a variety of ways for attaching motifs which react with the active site of a proteolytic enzyme, especially for aspartyl, metallo, serine and cysteinyl proteases, an enzyme inhibitor can be readily synthesised. Moreover, amide bond replacements or transition state mimetics can be incorporated into the molecule, which would be especially useful for the inhibition of aspartyl or metallo proteases.

The method described also facilitates the rapid development of a screening assay for novel protease



inhibitors. The most potent fluorogenic substrate discovered by library screening can subsequently be used for the detection of inhibitors of the particular proteolytic enzyme under scrutiny.

- 5     The presence of an inhibitor within the compound libraries described is readily detected by retreatment of the assay mixture with the most active fluorogenic substrate, which will allow the immediate measurement of the remaining proteolytic enzyme activity.
- 10    The invention provides self-decoding, combinatorial fluorogenic libraries, and it will greatly facilitate the design and invention of novel protease inhibitors because:
- i.     The peptides of the library may have increased aqueous solubility in comparison to peptides  
15       containing similar and other fluorogenic and quencher groups.
  - ii.    The peptides are stable to contaminating exopeptidases.
  - iii.   The self deconvolution method described, coupled with  
20       the continuous analysis of the rate of substrate cleavage data, allows the immediate identification of the most active binding motif contained within the substrate library.
  - iv.    The method allows for the rapid assessment of the  
25       enzyme assay mixture for any compounds in the library that are acting as enzyme inhibitors.

## Abbreviations

Abbreviations used herein are as follows:

Abbreviations for amino acids and nomenclature of peptide structures follow the recommendations given in: IUPAC-IUB Commission on Biochemical Nomenclature, (*J. Biol. Chem.*, **247**, 997, 1971). All chiral amino acids are of the L configuration unless otherwise stated. Other abbreviations used are :

-Abu ,  $\beta$ -amino butyric acid, : Abz, 2-amino benzoyl : ACH,  
10 1-amino-1-carboxy-cyclohexane : ACP, 1-amino-1-carboxy-  
cyclopropane : Bip, Biphenylalanine : n-Bu, n-  
butoxycarbonyl : Bz, Benzoyl : Bz(2-carboxy), 2-  
carboxybenzoyl : Bu<sup>t</sup>Gly, tert-Butylglycyl : BOP,  
benzotriazoyl-oxy-tris-(dimethylamino)-phosphonium  
15 hexafluorophosphate: Cha, cyclohexylalanine : Chex, 1-  
carboxycyclohexyl : eAHA, gamma aminohexanoyl : HBTU, O-  
benzotriazoyl-N,N,N',N'-tetramethyluronium  
hexafluorophosphate : HOBt, 1-hydroxybenzotriazole : Hyp,  
trans-4-hydroxyprolinyl : hLeu, homoleucyl : 2NaI, 2-  
20 naphthylalanine : NMM, N-methylmorpholine : Piv, pivoyl :  
3pyr, 3-pyridylalanine : Tic, 2-carboxytetrahydroquinolyl  
: Tyr(NO<sub>2</sub>), 3-nitrotyrosine.

DMF, dimethylformamide; Fmoc, fluorenylmethoxycarbonyl;  
HPLC, high performance liquid chromatography; Pfp,  
15 pentafluorophenyl, tBoc, tert-butoxycarbonyl; tBu, tert-  
butyl; TFA, trifluoroacetic acid; Pmc, pentamethyl  
chroman, Phf, pentamethylbenzofuran, TBTU, 2-(1H-

Benzotriazole-1-yl)-1,1,1,3,3-tetramethyluronium  
tetrafluoroborate; Trt, Trityl.

p.Aba, 4-aminobenzoyl; Aib, Aminoisobutyric acid; Bip,  
Biphenylalanine; nBu, n-Butyl; Bz, Benzoyl; Cmpi,  
5 Carboxymethylpiperazine; Deg, Diethylglycine; DIPEA, N,N-  
Diisopropyl-ethylamine; HATU, O-(7-azabenzotriazol-1-yl)-  
1,1,3,3-tetramethyluronium hexafluorophosphate; HOAT, 1-  
hydroxy-7-azabenzotriazole; Naph, Naphthylalanine; 3.Pyr,  
3-pyridylalanine; Tyr(NO<sub>2</sub>), 3-nitro-tyrosine.

## References

1. I. Schlechter and A. Berger, Biochem. Biophys. Res. Commun., 1967, 27, 157-162)
2. A. Carmel et al., FEBS Lett., 1973, 30, 11.
3. M. M. Meldal and I. Svendsen, J. Chem. Soc. Perkin Trans.1, 1995, 1591-1596,
4. M. Meldal and K. Breddam, Anal. Biochem., 1991, 195, 141-147,
5. T. Forster, Ann. Phys., 1948, 6, 55.
6. A. Yaron, A. Carmel, and E. Katchalski-Katzir, Anal. Biochem. 1979, 95, 228 and references therein.
7. S.A. Latt et al., Anal. Biochem., 1972, 50, 56.
8. A. Persson et al., Anal. Biochem., 1977, 83, 2,96.
9. I. Yu Filppova et al., Bioorg. Khim., 1986, 12, 1172.
10. J. Pohl et al., Anal. Biochem., 1987, 165, 96.
11. S.J. Pollack et al., J. Am. Chem. Soc., 1989, 111, 5961.
12. E. K. Bratovanova and D.D. Petkov, Analytical Biochem., 1987, 162, 213.
13. J. Singh et al., J. Med. Chem., 1995, 38, 217-219 and references therein.
14. M. Green et al. in 'Innovation and Perspectives in Solid Phase Synthesis' (R. Epton Ed.) Mayflower Worldwide Ltd., Birmingham, UK. 1994, 239-244.
15. J. R. Petithory et al., Proc. Natl. Acad. Sci. USA, 1991, 88, 11510-11514
16. J. Berman et al., J. Biol. Chem., 1992, 267, 1434-1437.
17. H. Drevin, A. -T. Martin, J. Carlsson, S. Oscarsson, T. Lovgren, I. Hemmila and M. Kwiatkowski, WO89/10975, (5-5-1988)

18. A. J. Garman and N. G. Phillips, WO 94/28166 (27-5-1993)
19. G.T. Wang and E.D. Matayoshi, E.P. 428000 (3-11-1989)
- 5 20. G. A. Krafft, G. T. Wang and E.D. Matayoshi, EP 428000, (3-11-1989).
21. G. R. Marshall and M. V. Toth, USP 5,164,300, (11-12-1990).
22. G. R. Marshall and M. V. Toth, USP 5,011,910, (28-12-1989).
- 10 23. K. T. Chapman, N. A. Thornberry, M. Maccoss, J. R. Weidner, R. A. Mumford, W. K. Hagmann, EP 528487A (16-8-1991)
24. R. P. Haugland, WO 93/04077, (23-8-1991)
- 15 25. R. M. Valerio, A. M. Bray N. J. Maeji, Int. J. Peptide Protein Res., 1994, 44, 158-165
26. M. Bastos, N. J. Maeji and R. H. Abeles, Proc. natl. Acad. Sci., 1995, 92, 6738-6742.
27. 'Solid Phase Peptide Synthesis', E. Atherton and R. C. Sheppard, IRL Press 1989.
- 20

Claims

1. A compound represented by the formula A-B-C-D-nE-F  
[I] in which;  
A represents a fluorescor internally quenched by F;  
5 B, C, D, and E represent groups such that the scissile  
bond between any two of these groups is a suitable bond;  
F represents a quencher capable of internally quenching  
the fluorescor A; and  
n represents an interger between 1 and 4 inclusive.
- 10 2. A compound according to claim 1 wherein A represents  
an unsubstituted or substituted anthranilic acid  
derivative.
3. A compound according to claim 1 or 2 wherein B, C, D  
and E independently represent natural or unnatural amino  
15 acid residues.
4. A compound according to any preceding claim wherein F  
represents an unsubstituted or substituted 3-nitrotyrosine  
derivative.
5. A compound according to any preceding claim wherein  
20 the scissile bond between D and E is an unsubstituted  
amide bond.
6. A compound according to any preceding claim wherein  
the formula further comprises G which ensures the compound  
is imparted with aqueous solubility.
- 25 7. A compound according to claim 6 which is represented  
by the formula A-B-C-D-n(E)-F-G in which

A, B, C, D, E, F and n are as defined in any one of claims 1 to 6; and

G represents a hydrophilic moiety which is not an enzyme substrate.

- 5     8.     A compound according to claim 6 or 7 in which G represents an aspartyl amide moiety.
9.     A combinatorial library of FRET compounds comprising a mixture of compounds of formula [I] of claim 1.
10.    A set of compounds which comprises two complementary  
10     FRET compound libraries.
11.    Apparatus which comprises an auto-deconvoluting set of compounds according to claim 10 that facilitates the invention of novel inhibitors of proteolytic enzymes and the rapid generation of structure-activity relationships  
15     (SAR) by the detection and measurement of proteolytic enzyme activity.
12.    Apparatus according to claim 11 wherein the set comprises a plurality of mixtures of FRET compounds according to claim 1.
13.    Apparatus according to claim 11 or 12 which comprises  
20     complementary compound libraries wherein any two FRET molecules in a single mixture of the first library are not found in a single mixture of the second library.

14. Apparatus according to any of claims 11 to 13 wherein the library comprises compounds of represented by the formula  $A-B_{1:n} - C_{m:n} - D_{p:n} - n (E_{1:n}) - F$  in which;  
A represents a fluorescor internally quenched by F;  
5 B, C, D and E represent groups such that the scissile bond between any two of these groups is an unsubstituted amide bond;  
F represents a quencher capable of internally quenching the fluorescor A; and  
10 n represents an integer between 1 and 4 inclusive.
15. Apparatus according to claim 14 wherein A represents an unsubstituted or substituted anthranilic acid derivative.
16. Apparatus according to claim 14 or 15 wherein B, C, D  
15 and E independently represent natural or unnatural amino acid residues.
17. Apparatus according to any one of claims 14 to 16 wherein F represents an unsubstituted or substituted 3-nitrotyrosine derivative.
- 20 18. Apparatus according to any one of claims 14 to 17 wherein the scissile bond between D and E is an unsubstituted amide bond.
19. Apparatus according to any one of claims 14 to 18 wherein the formula further comprises G which ensures that  
25 compounds in the library are imparted with aqueous solubility.



20. Apparatus according to any one of claims 14 to 19 wherein the library comprises mixtures of compounds represented by the formula  $A-B_{1:n} - C_{1:n} - D_{1:n} - n(E_{1:n}) - F-G$  in which

5 A, B, C, D, E, F and n are as defined in any one of claims 12 to 16 and

G represents n a hydrophilic moiety which is not an enzyme substrate.

21. Apparatus according to claim 19 or 20 in which G  
10 represents an aspartyl amide moiety.

22. Apparatus according to any one of claims 11 to 21 wherein the library comprises 1600n compounds as 80n mixtures of 20 distinct, identifiable compounds.

23. Apparatus according to claim 22 wherein the mixtures  
15 of 20 distinct, identifiable compounds are placed separately into each of 80 wells of an microtitre plate.

24. Use of a combinatorial FRET library in a method which provides rapid generation of structure-activity relationships (SAR) which comprises detection and  
20 measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules to find a substrate or substrates for the enzyme.

25. Use of a combinatorial FRET library in a method for

26. A method of identifying and synthesising an inhibitor of a proteolytic enzyme which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules, deconvoluting the library to find a substrate or substrates for the enzyme and synthesis of an inhibitor based on the substrate or substrates.

27. An inhibition assay which uses a FRET molecule, which has been identified as a substrate for the enzyme, wherein the molecule is assayed with the enzyme separately against a panel of possible inhibitors.

28. A method which provides the rapid generation of structure-activity relationships (SAR) which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules to find a substrate or substrates for the enzyme.

29. A method which comprises the identification of an enzyme inhibitor or inhibitors wherein a FRET compound which has been identified as a substrate is used in an inhibition assay with the enzyme separately against a panel of possible inhibitors.

30. A method according to claim 29 wherein the assay is performed by using the apparatus according to any of claims 11 to 23.

31. A method according to any preceding method claim wherein the compounds of the library are synthesized using a solid phase technique.

32. A complementary pair of compound libraries L1 and L2  
5 which constitute a set containing compounds of formula:

Aa-Bb-Cc-Dd-n(Ee)-Ff-Gg

giving  $a \times b \times c \times d \times e \times f \times g = M_n$  compounds in each library, there being a predetermined number (P1, P2) of mixtures each consisting of a predetermined number (Q1,  
10 Q2) of individual identifiable compounds in each library, wherein both L1 and L2 contain the same  $M_n$  compounds, but wherein any two compounds which are found together in one mixture of Q1 compounds of L1 are not found together in any one of the P2 mixtures of L2.

15 33. A method of screening for enzymic activity using the libraries L1, L2 according to claim 32 in which the P1 mixtures of L1 and the P2 mixtures of L2 are each placed separately into individual wells of two well plates, the well plates having wells arranged in a format adapted to  
20 allow deduction of a unique active compound formula from the presence of activity in one well of the well plate of L1 and one well of the well plate of L2.

34. A compound, apparatus, method or assay as substantially described herein with reference to example



116

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10		
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10		
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10		
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10		
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10		
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10		
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10		
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10		

Figure 1



2/16

First Example of Library Matrix where  $n=1$

Component Distribution in Plate 1, Library 1 ( $n=1$ )

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>
D2	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>
D3	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>
D4	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>
D5	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>
D6	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>
D7	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>
D8	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>	B <sub>1,10</sub> E <sub>1,2</sub>

Figure 2





3/16

Component Location in Plate 1, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C <sub>1,1,1</sub>	C <sub>1,1,2</sub>	C <sub>1,1,3</sub>	C <sub>1,1,4</sub>	C <sub>1,1,5</sub>	C <sub>1,1,6</sub>	C <sub>1,1,7</sub>	C <sub>1,1,8</sub>	C <sub>1,1,9</sub>	C <sub>1,1,10</sub>
	D <sub>1,1,1</sub>	D <sub>1,1,2</sub>	D <sub>1,1,3</sub>	D <sub>1,1,4</sub>	D <sub>1,1,5</sub>	D <sub>1,1,6</sub>	D <sub>1,1,7</sub>	D <sub>1,1,8</sub>	D <sub>1,1,9</sub>	D <sub>1,1,10</sub>
E2	C <sub>1,2,1</sub>	C <sub>1,2,2</sub>	C <sub>1,2,3</sub>	C <sub>1,2,4</sub>	C <sub>1,2,5</sub>	C <sub>1,2,6</sub>	C <sub>1,2,7</sub>	C <sub>1,2,8</sub>	C <sub>1,2,9</sub>	C <sub>1,2,10</sub>
	D <sub>1,2,1</sub>	D <sub>1,2,2</sub>	D <sub>1,2,3</sub>	D <sub>1,2,4</sub>	D <sub>1,2,5</sub>	D <sub>1,2,6</sub>	D <sub>1,2,7</sub>	D <sub>1,2,8</sub>	D <sub>1,2,9</sub>	D <sub>1,2,10</sub>

Figure 3



4/16

Component Location in Plate 2, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>
E2	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>

Figure 4



5/16

Component Location in Plate 3, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>
E2	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>

Figure 5



6/16

Component Location in Plate 4, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>
	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>
E2	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>	C <sub>1,10</sub>
	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>	D <sub>1,10</sub>

Figure 6





7/16

Example Library where n=4

Component Distribution in Plate 1, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>
D2	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>
D3	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>
D4	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>
D5	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>
D6	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>
D7	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>
D8	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>	B <sub>1,1,1,0</sub> E <sub>1,1,2</sub>

Figure 7



8/16

Component Location in Plate 2, Library 1 n=4

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>
D2	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>
D3	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>
D4	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>
D5	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>
D6	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>
D7	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>
D8	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>	B <sub>1-10</sub> E <sub>3-4</sub>

Figure 8



9/16

Component Location in Plate 3, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$
D2	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$
D3	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$
D4	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$
D5	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$
D6	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$
D7	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$
D8	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$	$B_{1,1,1,0}$ $B_{5,5,5,5}$

Figure 9



10/16

Component Location in Plate 4, Library 1 (n=4).

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>
D2	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>
D3	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>
D4	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>
D5	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>
D6	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>
D7	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>
D8	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>	B <sub>1,10</sub> E <sub>1,3</sub>

Figure 10





11/16

Component Location in Plate 1, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>
E2	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>
E3	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>
E4	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>
E5	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>
E6	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>
E7	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>
E8	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>	C <sub>1-10</sub> D <sub>1-2</sub>

Figure 11.



12/16

Component Location in Plate 2, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>
E2	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>
E3	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>
E4	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>
E5	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>
E6	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>
E7	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>
E8	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>	C <sub>1-10</sub> D <sub>3-4</sub>

Figure 12



13/16

Component Location in Plate 3, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>
E2	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>
E3	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>
E4	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>
E5	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>
E6	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>
E7	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>
E8	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>	C <sub>1-10</sub> D <sub>5-6</sub>

Figure 13



14/16

Component Location in Plate 4, Library 2 (n=4).

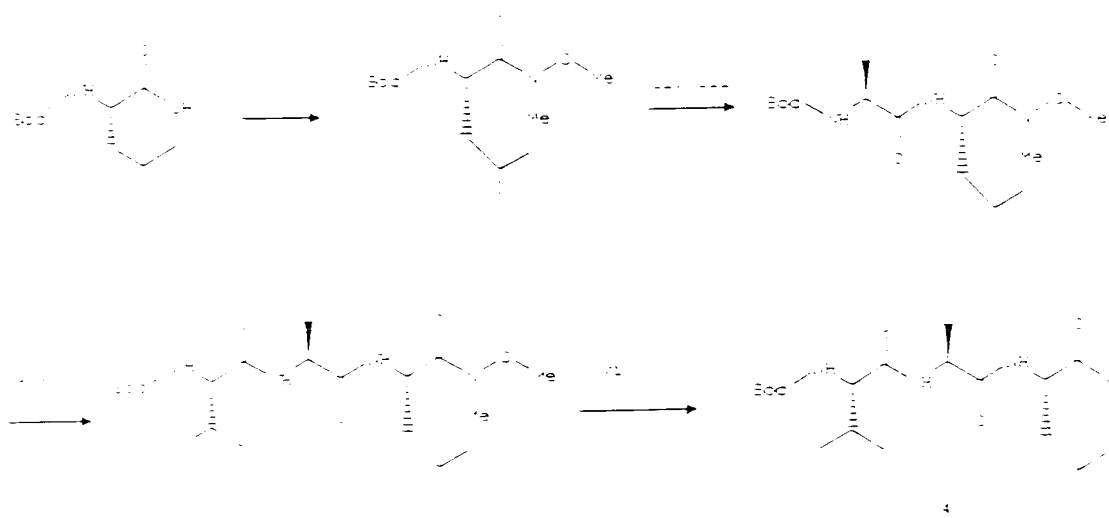
	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>
E2	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>
E3	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>
E4	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>
E5	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>
E6	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>
E7	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>
E8	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>	C <sub>1,10</sub> D <sub>7,8</sub>

Figure 14.





15/16

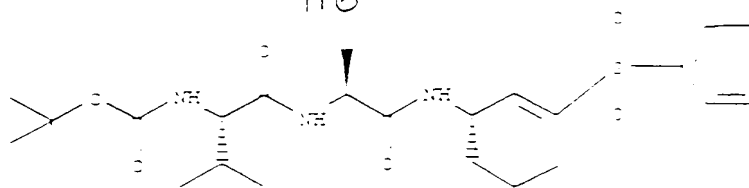


1. 1-methyl-2-norbornene, then 1,3-dinitro-2-norbornene, DNF, 100%  
2. 1-methyl-2-norbornene, then 1,3-dinitro-2-norbornene, DNF, 100%  
3. 1-methyl-2-norbornene, then 1,3-dinitro-2-norbornene, DNF, 100%

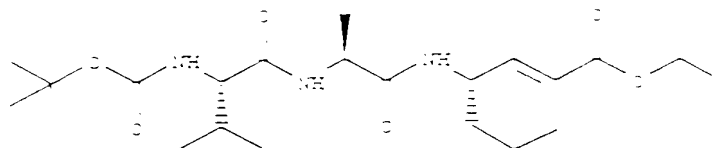
Figure 15



16/16



1a



1b

Figure 16

1. The first part of the document

is a list of the names of the

persons who

are mentioned in the